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Enhanced Cytotoxicity through Conjugation of a "Clickable" Luminescent Re(I) Complex to a Cell-Penetrating Lipopeptide

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Abstract: Re(I) tricarbonyl polypyridine-based complexes are particularly attractive metal complexes in the field of inorganic chemical biology due to their luminescent properties, ease of conjugation to targeting biomolecules, and the possibility to prepare their "hot" (^{99m}Tc) analogues for radioimaging. In this study, we prepared and characterized a novel, "clickable" complex, [Re(2,2'-bipyridine)(3-ethynylpyridine)(CO)₃](BF₄) ([Re(CO)₃(bipy)(py-alkyne)](BF₄)), exhibiting the characteristic luminescent properties and moderate cytotoxicity of this general class of compound. Using Cu(I)-catalyzed "click" chemistry, the complex was efficiently attached to a lipidated peptide known to increase cell permeability, namely, the myristoylated HIV-1 Tat peptide (myr-Tat), to give Re-myr-Tat. Fluorescence microscopy localization in human cervical cancer cells (HeLa) confirmed enhanced cellular uptake of Re-myr-Tat compared with [Re(CO)₃(bipy)(py-alkyne)](BF₄), and cytotoxicity studies showed that this resulted in an increase in potency to a level comparable with cisplatin (13.0 ± 2.0 M).

DOI: <https://doi.org/10.1021/ml500158w>

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ZORA URL: <https://doi.org/10.5167/uzh-98805>

Journal Article

Accepted Version

Originally published at:

Leonidova, Anna; Pierroz, Vanessa; Adams, Luke A; Barlow, Nicholas; Ferrari, Stefano; Graham, Bim; Gasser, Gilles (2014). Enhanced Cytotoxicity through Conjugation of a "Clickable" Luminescent Re(I) Complex to a Cell-Penetrating Lipopeptide. *ACS Medicinal Chemistry Letters*, 5(7):809-814.

DOI: <https://doi.org/10.1021/ml500158w>

Enhanced Cytotoxicity through conjugation of a “clickable” luminescent Re(I) complex to a cell-penetrating lipopeptide

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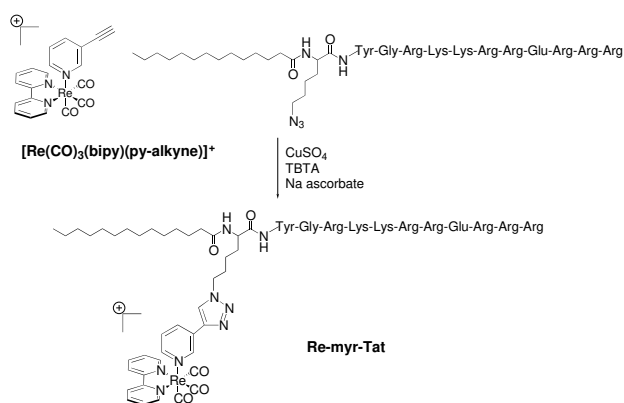
Anticancer, Fluorescence Microscopy, Medicinal Organometallic Chemistry, Peptide, Rhenium Complexes

ABSTRACT: Re(I) tricarbonyl polypyridine-based complexes are particularly attractive metal complexes in the field of inorganic chemical biology due to their luminescent properties, ease of conjugation to targeting biomolecules and the possibility to prepare their “hot” ^{99m}Tc analogs for radioimaging. In this study, we prepared and characterized a novel, “clickable” complex, [Re(2,2'-bipyridine)(3-ethynylpyridine)(CO)₃](BF₄) ([Re(CO)₃(bipy)(py-alkyne)](BF₄)), exhibiting the characteristic luminescent properties and moderate cytotoxicity of this general class of compound. Using Cu(I)-catalyzed “click” chemistry, the complex was efficiently attached to a lipidated peptide known to increase cell permeability, namely the myristoylated HIV-1 Tat peptide (**myr-Tat**), to give **Re-myr-tat**. Fluorescence microscopy localization in human cervical cancer cells (HeLa) confirmed enhanced cellular uptake of **Re-myr-Tat** compared with [Re(CO)₃(bipy)(py-alkyne)](BF₄), and cytotoxicity studies showed that this resulted in an increase in potency to a level comparable with cisplatin (13.0 ± 2.0 μM).

Re(I) tricarbonyl [2 + 1] complexes based on polypyridine-derived ligands, such as (Re(bipy)(L)(CO)₃) (L = monodentate ligand), have attracted considerable attention in the last decade as catalysts,¹ photosensitizers in photocatalytic water reduction^{2,3} and CO-releasing molecules.⁴ They remain, however, best known for their outstanding photochemical properties (large Stokes shifts, long emission lifetimes, resistance to photobleaching),⁵ which make them excellent candidates for cellular imaging and ion sensing applications.⁶⁻¹⁴ Their use in these areas has been spurred on by their good biocompatibility¹⁵ and the possibility to incorporate targeting vectors *via* the pyridine or bipyridine-based ligands.^{9, 16, 17} Significantly, the “hot” ^{99m}Tc analogs of these Re compounds could also be prepared, making them promising multimodal agents.^{16, 18-20}

While Re(I) tricarbonyl polypyridine-based complexes are normally only moderately toxic, or essentially non-toxic, several other Re(I) compounds have been reported to be as active or even more potent than cisplatin.^{8, 21-28} Given how active many organometallic compounds are known to be against cancer cells lines,²⁹⁻³⁴ it is surprising that the cytotoxic potential of these Re(I) complexes has not yet been fully explored, especially in light of their aforementioned advantages.

In this work, we aimed to improve the cytotoxicity of a Re(I) compound, namely [Re(CO)₃(bipy)(py-alkyne)]⁺, whose moderate cytotoxicity was discovered by serendipity in the course of a separate study. To do this, we envisaged enhancing cellular uptake of the complex *via* conjugation to a myristoylated HIV-1



Scheme 1. Bioconjugation of [Re(CO)₃(bipy)(py-alkyne)]⁺ to a myristoylated Tat peptide.

Tat peptide (Scheme 1). HIV-1 Tat is a membrane translocation sequence from HIV, while myristic acid is a saturated linear fatty acid that naturally occurs as a post-translational protein modification. Both myristic acid and myristoylated Tat peptide were shown to increase the cellular uptake of compounds when conjugated to them.^{35, 36} In this study, the Re complex was appended to an azide-modified myristoylated Tat peptide *via* “click” chemistry. Over the past few years, click chemistry has been successfully employed to couple other organometallic compounds to peptides, either by solid-phase or solution-phase methods.³⁷⁻⁴⁰

$[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ was synthesized following an established literature procedure employed for $\text{Re}(\text{I})$ fac-tricarbonyl bipyridyl complexes with a substituted pyridine ligand.⁴¹ In brief, the initially formed Re tricarbonyl bipyridyl chloride complex was activated *via* halide abstraction as the corresponding acetonitrile complex. The acetonitrile ligand was then displaced by 3-ethynylpyridine. The formation and purity of the desired product was confirmed by ^1H - and ^{13}C -NMR, HR-MS (Figures S1–3) and elemental analysis. Of note, loss of the monodentate pyridine ligand (m/z 427.0 $[\text{M-py}]^+$) during ionization was observed in the MS spectrum (Figure S3). $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ exhibits the typical photophysical properties for this type of $\text{Re}(\text{I})$ complex.^{10, 17, 25, 42–50} The UV-vis absorption spectra showed intense bands at 250–330 nm (absorption coefficients around $15,000 \text{ M}^{-1}\text{cm}^{-1}$) and a weaker shoulder at 350–360 nm (about $4,500 \text{ M}^{-1}\text{cm}^{-1}$) (Figure S5). The former are normally attributed to spin allowed intraligand transitions ($^1\text{IL} = (\pi \rightarrow \pi^*)$ (bipyridine and pyridine), while the latter is assigned to spin-allowed metal-to-ligand charge transfer ($^1\text{MLCT}$) [$d\pi(\text{Re}) \rightarrow \pi^*(\text{bipy})$]. Upon irradiation in the MLCT band (355 nm), $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ displayed a strong emission centered at 550 nm (Figure S6). Long, sub-microsecond luminescence lifetimes (Table 1) and large Stokes shift indicate the phosphorescent nature of the emission,⁵¹ which can be ascribed to a triplet $^3\text{MLCT}$ [$d\pi(\text{Re}) \rightarrow \pi^*(\text{bipy})$] transition, as observed for similar complexes.^{4, 12} Typical emission lifetimes and quantum yields of $\text{Re}(\text{CO})_3(\text{bipy})(\text{L})$ complexes encompass a relatively broad range (0.05–9.6 μs and 0.002–0.59, respectively).⁵² The values measured for $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ are, in fact, very close to those of structurally similar $\text{Re}(\text{CO})_3(\text{bipy})(\text{L})$ complexes, such as isothiocyanate-substituted pyridine complexes.¹⁷ Of note, shorter lifetimes in water compared to acetonitrile can be explained by the greater polarity of water, which stabilizes the $^3\text{MLCT}$ excited state, thus lowering its energy and facilitating non-radiative processes.^{53, 54} The complex itself can be solubilized in pure water up to $0.79 \pm 0.13 \text{ mM}$ (25 °C).

Table 1. Emission lifetimes and quantum yields of $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$

Solvent	Aerated	Degased	Quantum yields (aerated)
H ₂ O	117 \pm 2 ns	128 \pm 3 ns	0.0048 \pm 0.0005
Acetonitrile	168 \pm 6 ns	329 \pm 1 ns	0.011 \pm 0.001

Since some studies have previously noted the loss of the monodentate pyridine ligand in solution for this type of complex,^{16, 18–20} the stability of $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ in water and human blood plasma was assessed. Following an experimental procedure similar to that previously reported by our group,^{28, 55–57} the complex and diazepam (used as an internal standard due to its known stability in blood plasma and water) were incubated in human plasma or double distilled water for up to 72 h. The aqueous phase was then extracted with dichloromethane and the organic phase analyzed by UPLC-MS. Two peaks corresponding to $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})]^+$ (1.7 min, m/z 530.1 $[\text{M}]^+$, 427.1 $[\text{M-ligand}]^+$) and diazepam (2.1 min, m/z 285.2 $[\text{M+H}]^+$) could be

clearly identified (Figure S9–12). The percentage of decomposed $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})]^+$ was then calculated using diazepam as the internal standard. As shown on Figure 1, decomposition proceeded significantly faster in human blood

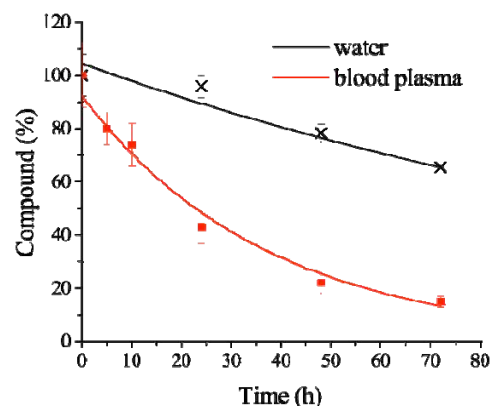


Figure 1. $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})]^+$ stability in human blood plasma and water (double distilled) at 37 °C.

plasma (half-life of approx. 22 h) than pure water (half-life of approx. 5 days), probably due to the substitution of the pyridine ligand by stronger donor groups present in blood plasma proteins (e.g. histidine or cysteine). These results are consistent with the recent study by the Valliant group, which showed a marked dependence of the pyridine ligand lability on its basicity/leaving group ability.¹⁶ The plasma stability of $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ is on par with the most stable compounds reported by Valliant and co-workers.¹⁶

The azide-modified myristoylated Tat peptide (**myr-Tat**) was prepared *via* standard solid-phase peptide synthesis techniques and then purified by preparative HPLC to give a sticky yellow solid, which was unambiguously characterized by HR-MS and HPLC (Figures S13–14). It was then successfully conjugated to $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ *via* Cu(I)-catalyzed click chemistry using similar experimental conditions to those reported by Fokin and co-workers.⁵⁸ The crude compound was purified by preparative HPLC to yield **Re-myr-Tat** as a pale yellow solid, which was characterized by UPLC-MS and MALDI-TOF (Figures S15–16). A single peak was observed in the UPLC-MS chromatogram and – consistent with the MS spectrum of complex – both the desired product and fragments resulting from pyridine ligand dissociation appeared in the MS spectrum (m/z 338.9 $[\text{M-Re}(\text{CO})_3(\text{bipy})+6\text{H}]^{6+}$, 406.4 $[\text{M-Re}(\text{CO})_3(\text{bipy})+5\text{H}]^{5+}$, 491.6 $[\text{M+4H}]^{5+}$, 507.8 $[\text{M-Re}(\text{CO})_3(\text{bipy})+4\text{H}]^{4+}$, 614.2 $[\text{M+3H}]^{4+}$, 818.7 $[\text{M+2H}]^{3+}$). In the MALDI-TOF spectrum, only the fragmented product (m/z 2026.4 $[\text{M-Re}(\text{CO})_3(\text{bipy})+\text{H}]^+$) was detected due to the harsher ionization conditions compared to ESI-MS. The presence of the *fac*- $\text{Re}(\text{CO})_3$ core in the **Re-myr-Tat** bioconjugate was unambiguously confirmed by the presence of the CO stretching bands in the IR spectrum, which appeared in the same range to that of $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ (Figure S17). The influence of the **myr-Tat** moiety on the lipophilicity of the resulting bioconjugate **Re-myr-Tat** was evaluated by measuring the distribution coefficient between octanol and

phosphate buffer, pH 7.01 ($\log D_{7.01}$), using a similar procedure to one used by our group for Ru complexes.⁵⁹ Interestingly, although **myr-Tat** contains both a long lipophilic fatty acid chain and a highly positively-charged peptide sequence, the net effect is an increase in lipophilicity, namely from a $\log D_{7.01}$ of -0.36 ± 0.05 for $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})]^+$ to 0.86 ± 0.15 for **Re-myr-Tat**. This change could potentially improve the cellular uptake of **Re-**

(Figure S18). However, the cells appeared to be already slightly affected by the conjugate at this concentration, so this change in localization could be due to toxin stress.

Next, the cytotoxicity of $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ and **Re-myr-Tat** towards HeLa cells was evaluated by incubating the cells with increasing concentrations of the compounds for 48 h and quantifying cell viability using the resazurin assay.⁶⁶ The

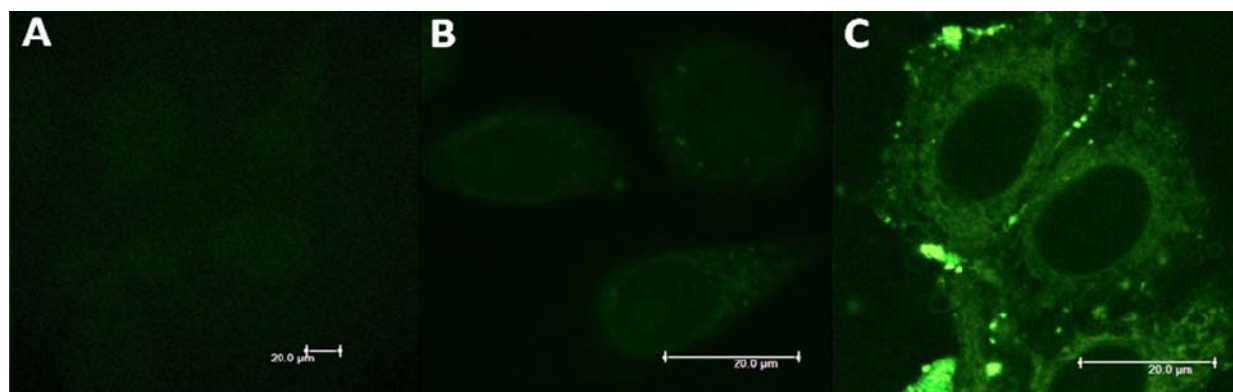


Figure 2. Fluorescence microscopy of HeLa cells fixed after 2 h: A) untreated cells; B) treated with $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ at 100 μM ; C) treated with **Re-myr-Tat** at 20 μM .

myr-Tat, as higher lipophilicity can favor the entry of compounds into cells, and hence enhance the cytotoxicity.⁵⁹⁻⁶³

With the desired organometallic complex and bioconjugate in hand, we investigated the intracellular fate of both compounds in human cervical cancer cells (HeLa) by fluorescence microscopy. The cells were first incubated for 2 h with an appropriate concentration of each compound, then fixed with formaldehyde and finally imaged. Figure 2 shows a pronounced difference in emission intensity between the cells treated with $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ (B) or **Re-myr-Tat** (C). Indeed, while the concentration of **Re-myr-Tat** in the cell medium was five times lower than that of the complex, the cells incubated with **Re-myr-Tat** appeared much brighter, indicating significantly higher uptake (it is important to keep in mind that the intensity of the emission signal of a compound in the cells sometimes fails to correlate with uptake, as luminescence can be quenched in the cellular environment).^{28, 64} In terms of subcellular localization, the complex and the bioconjugate were visualized in the cytoplasm (although weak signals were sometimes detected in the nucleoli of cells incubated with the complex) – a localization typically observed for this type of Re complex.^{10, 12, 15, 16} Of note, whilst the HIV-1 Tat peptide sequence has been reported to promote nuclear localization,⁶⁵ with a myristoylated Tat derivative dual-labeled with Gd-DOTA and fluorescein previously shown to stain both nucleoli and nuclear membranes,³⁶ this was not the case for **Re-myr-Tat**. Our microscopy images were recorded at a relatively low **Re-myr-Tat** concentration that left most of the cells alive after 2 h. The Gd-DOTA myr-Tat derivative, on the other hand, was imaged at a much higher concentration (260 μM), at which about 60% of the cells were already dying.³⁶ When HeLa cells were incubated with a higher concentration (2.5-fold increase) of **Re-myr-Tat**, localization in nuclear membrane and nucleoli was also observed

toxicity of our compounds was compared with that of an established metal-containing anti-cancer drug, namely cisplatin. Although several studies report on the cytotoxicity of Re(I) tricarbonyl bipyridine-pyridine complexes, their anti-proliferative effect has generally been found to range from non-existent to moderate; $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$ does not deviate from this trend (Table 2). A series of similar complexes, namely $\text{Re}(\text{phen})(\text{diaminopy})(\text{CO})_3$, display IC_{50} values 2–3-fold higher than cisplatin.²⁵ Significantly, however, the coupling of $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})]^+$ to the myristoylated Tat peptide was found to significantly increase the cytotoxicity of the resulting bioconjugate, bringing it on par with cisplatin.

Table 2. Cytotoxicity data (IC_{50} values) for $[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})](\text{BF}_4)$, **Re-myr-Tat and cisplatin towards HeLa cells.**

Compounds	HeLa IC_{50} (μM)
$[\text{Re}(\text{CO})_3(\text{bipy})(\text{py-alkyne})]^+$	29.9 ± 6.1
Re-myr-Tat	13.0 ± 2.0
Cisplatin	9.1 ± 2.8

* Experiments were performed in triplicates.

In conclusion, in this work, we prepared and characterized a new “clickable” Re(I) complex, which could be successfully conjugated to an azide-containing myristoylated Tat peptide to give **Re-myr-Tat**. Cytotoxicity and biological studies with HeLa cells revealed that the anti-proliferative effect of the complex could be enhanced considerably by the addition of a cell uptake-enhancing biomolecule.

ASSOCIATED CONTENT

Supporting Information. Electronic Supplementary Information (ESI) available: characterization of the compounds (NMR, MS, UPLC-MS, UV, emission and IR spectra), emission quantum yields measurements, additional information on stability measurements (UPLC-MS spectra). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ACKNOWLEDGMENT

This work was supported by the Swiss National Science Foundation (Professorship N° PP00P2_133568 and Research Grants N° 200021_129910 and N° 200020_146776 to G.G.), the University of Zurich (G.G and S.F.), the Stiftung für wissenschaftliche Forschung of the University of Zurich (G.G and S.F.), the Stiftung zur Krebsbekämpfung (S.F), the Huggenberger-Bischoff Stiftung (S.F), the University of Zurich Priority Program (S.F.), and the Australian Research Council through the award of a Future Fellowship to B.G. The authors thank the Center for Microscopy and Image Analysis (University of Zurich).

ABBREVIATIONS

Bipy – bipyridine
DOTA – 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid
ESI – electrospray ionization
HeLa – cervical cancer cell line
HIV – human immunodeficiency virus
HPLC – high-performance liquid chromatography
L – ligand
NMR – nuclear magnetic resonance
HR-MS – high resolution mass spectroscopy
MALDI-TOF – matrix-assisted laser desorption ionization time-of-flight
MLCT – metal-to-ligand charge transfer
Py – pyridine
Tat – “trans-activator of transcription”, here a cell-permeating peptide sequence derived from HIV-1 Tat protein
UPLC – ultra-performance liquid chromatography
UV-vis – ultra-violet-visible light

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